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Disruption of the developmental programme of *Trypanosoma brucei* by genetic ablation of TbZFP1, a differentiation-enriched CCCH protein

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Summary

The regulation of differentiation is particularly important in microbial eukaryotes that inhabit multiple environments. The parasite *Trypanosoma brucei* is an extreme example of this, requiring exquisite gene regulation during transmission from mammals to the tsetse fly vector. Unusually, trypanosomes rely almost exclusively on post-transcriptional mechanisms for regulated gene expression. Hence, RNA binding proteins are potentially of great significance in controlling stage-regulated processes. We have previously identified TbZFP1 as a trypanosome molecule transiently enriched during differentiation to tsetse midgut procyclic forms. This small protein (101 amino acids) contains the unusual CCCH zinc finger, an RNA binding motif. Here, we show that genetic ablation of TbZFP1 compromises repositioning of the mitochondrial genome, a specific event in the strictly regulated differentiation programme. Despite this, other events that occur both before and after this remain intact. Significantly, this phenotype correlates with the TbZFP1 expression profile during differentiation. This is the first genetic disruption of a developmental regulator in *T. brucei*. It demonstrates that programmed events in parasite development can be uncoupled at the molecular level. It also further supports the importance of CCCH proteins in key aspects of trypanosome cell function.

Introduction

Trypanosoma brucei spp. are protozoan parasites whose molecular cell biology has been studied extensively for two principal reasons. First, these organisms cause African sleeping sickness in humans and nagana in cattle, each of which imposes significant restriction on human health and prosperity in affected regions (http://www.who.int/topics/trypanosomiasis_african/en/). Also these parasites diverged very early from the main eukaryotic evolutionary lineage (Sogin *et al.*, 1986), making them an interesting model for the study of both fundamental eukaryotic processes and for highly specialist host-parasite adaptations. Trypanosomes have strict mechanisms facilitating survival in the mammalian host and their tsetse fly vector. For example, bloodstream forms evade immune responses by

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expression of a variable surface glycoprotein (VSG) coat, whereas tsetse-midgut procyclic forms express a procyclic-specific surface coat composed of EP and GPEET procyclin (Matthews *et al.*, 2004). In addition to surface antigen exchange, metabolic differentiation occurs, the bloodstream forms relying on glycolysis whereas the procyclic forms elaborate the mitochondrion to utilize proline as an energy source in the tsetse midgut. Morphologically the different forms of the parasite are clearly distinguishable and single copy organelles are maintained with a defined stage-regulated position within the cell (McKean, 2003). In particular, the kinetoplast lies at a posterior position in bloodstream forms, whereas it lies midway between the nucleus and posterior in procyclic forms. Cell differentiation is therefore an essential part of adaptation to the very different environments in which the parasite must proliferate during its life cycle.

Although considerable effort has gone into defining the events of differentiation (Roditi *et al.*, 1989; Ziegelbauer and Overath, 1990; Matthews and Gull, 1994; 1997), and establishing the temporal order in which they occur (Matthews *et al.*, 1995; Matthews and Gull, 1997; Matthews, 1999), little is known regarding the molecular controls that underlie these processes. In almost all eukaryotes the initiation of transcription is used as a major control point for differential gene expression. In contrast, trypanosomes have largely abandoned regulation of RNA polymerase II dependent transcription as a control mechanism. Instead these organisms rely on regulation at the post-transcription level in order to control cell function. Consequently molecules containing motifs able to bind RNA have the potential to function as *trans*-acting factors controlling developmental gene expression.

The CCCH zinc finger motif is found in proteins known to bind RNA (Lai *et al.*, 1999; Lai *et al.*, 2000; Hudson *et al.*, 2004) and regulate a broad range of cell functions (Bai and Tolias, 1996; Carballo *et al.*, 1998; Gao *et al.*, 2002) in several diverse organisms including *T. brucei* (Thompson *et al.*, 1996; De *et al.*, 1999; Kronnie *et al.*, 1999; Tabara *et al.*, 1999; Reese *et al.*, 2000; Hendriks *et al.*, 2003). Previously we identified a small protein, TbZFP1, that contained a CCCH zinc finger motif and whose expression was elevated during the early stages of differentiation (Hendriks *et al.*, 2001). A second, constitutively expressed, small CCCH protein TbZFP2 was also characterized and implicated in the initiation of differentiation, because RNAi for this molecule inhibited the process. Moreover ectopic overexpression of this molecule generated a procyclic–specific morphological phenotype, ‘nozzle’ formation, entailing posterior extension of the microtubule corset of the procyclic trypanosome cell. These features suggested that small CCCH proteins might be contributing to the regulation of trypanosome differentiation and morphogenesis.

In this study we show that TbZFP1 is developmentally regulated such that expression, even outwith the normal genomic environment, is post-transcriptionally downregulated in bloodstream form *T. brucei*. Moreover, TbZFP1 bloodstream stage null mutants were unable to complete a specific event in differentiation to procyclic forms, repositioning of their mitochondrial genome (kinetoplast). Analyses of these mutants furthermore indicated that the control of trypanosome development is not under strictly hierarchical control, with events before and after this morphological restructuring being unaffected. These results emphasize the link between cellular architecture and life cycle development, and further establish a role for CCCH zinc finger proteins in controlling the events of trypanosome differentiation.

Results

TbZFP1 expression is strictly life cycle stage specific

TbZFP1 was initially isolated as an mRNA expressed at low levels in bloodstream form *T. brucei*, but transiently enriched during differentiation and maintained in the insect procyclic

form (Hendriks *et al.*, 2001). Protein expression could not be detected in cultured bloodstream forms (monomorphs) or in slender bloodstream forms (derived from rodent infections) (E.F. Hendriks and K.R. Matthews, unpubl. obs.) but low levels were detectable in bloodstream stumpy forms. Higher protein levels were observed transiently during differentiation and expression was maintained in procyclic forms (Hendriks *et al.*, 2001). Therefore, TbZFP1 mRNA and protein levels essentially correlate in differentiating cells and procyclic forms, whereas in bloodstream forms translational control may play a role. To investigate the regulation of TbZFP1 and assess the consequences of perturbing its normal expression, ectopic overexpression of this molecule was attempted in cultured monomorphic bloodstream forms and procyclic forms. Thus, a copy of TbZFP1 was generated that was modified to encode a protein with a C-terminal Ty1 epitope tag (Bastin *et al.*, 1996) and this cloned into the tetracycline inducible trypanosome expression vector pHD451 (Wirtz *et al.*, 1999). Figure 1A confirms that the C-terminally tagged TbZFP1 was significantly overexpressed in procyclic cells with respect to endogenous levels. When compared with the wild-type parental cell lines, or uninduced controls, in terms of growth, cell cycle status and morphology, however, no phenotype was detected (data not shown). Although transfected with the same construct, cultured bloodstream lines showed no detectable ectopic TbZFP1 protein whether assayed with the Ty1-specific antibody BB2 (Fig. 1B), or the TbZFP1-specific anti-peptide antibody (data not shown). Nonetheless, analysis by Northern blotting clearly showed the presence of a transcript derived from the TbZFP1 transgene in these cells (Fig. 1C; the transgene mRNA is of the predicted size and considerably shorter than the endogenous TbZFP1 mRNA resulting from the use of 3′ and 5′ untranslated regions in the expression construct that differ from those flanking the genomic copy of TbZFP1). Therefore in procyclic cells, where TbZFP1 is normally expressed, ectopic overexpression generates no detectable phenotype. However, in bloodstream cells endogenous or ectopically expressed TbZFP1 mRNA does not generate detectable protein, indicating that TbZFP1 protein levels are strictly regulated during the life cycle.

TbZFP1 is non-essential in bloodstream T. brucei *cells*

TbZFP1 gene silencing using RNA interference (RNAi) proved unsuccessful in procyclic and bloodstream cells (Hendriks *et al.*, 2001). Specifically, cell lines could be generated which expressed dsRNA for TbZFP1 under tetracycline induction, but this did not result in ablation of the endogenous transcript. Therefore, to investigate the requirement for TbZFP1 in procyclic forms and bloodstream forms we attempted to create a null mutant for TbZFP1 in each life cycle stage. Although single knockouts were readily generated using constructs targeting the first allele, procyclic forms deleted for the second allele could not be generated, despite using targeting constructs that were able to function when transfected independently. This suggested that TbZFP1 null mutants were not viable as procyclic forms. Supporting this, we were able to delete both TbZFP1 alleles in procyclic forms expressing a tetracycline regulatable ectopic rescue copy. Unfortunately, however, when expression of the ectopic copy was switched off by growth in the absence of tetracycline, significant leaky expression of ectopic TbZFP1 remained (data not shown). This precluded any phenotypic analysis of the cell lines although collectively these experiments suggest TbZFP1 has some essential function in procyclic form *T. brucei.*

Although informative TbZFP1 null mutants could not be generated as procyclic forms, we expected these to be viable in bloodstream forms where TbZFP1 is not expressed. Thus, to generate this line, a simple nested gene knockout approach was followed with the first TbZFP1 allele being replaced by a neomycin resistance cassette, also encoding a copy of the tetracycline repressor (Fig. 2A). This facilitated introduction of a tetracycline inducible rescue copy of TbZFP1 (see later) either before or after the second allele of the gene had been replaced with a hygromycin resistance cassette.

Several bloodstream transfectants were obtained for the first allele knockout using the Neomycin selectable marker. Eight independent Δzfp1::NEO cell lines were pooled and transfected with a construct conferring resistance to hygromycin and targeted to the second TbZFP1 allele. The resulting transfectants (Δzfp1::NEO/Δzfp1::HYG) were then cloned by limiting dilution. Two clones were analysed for the expression of TbZFP1 mRNA (one is shown in Fig. 2B). This revealed that each null mutant cell line expressed no TbZFP1 mRNA, whereas the wild-type and single knockout cell lines contained detectable levels of TbZFP1 mRNA (Fig. 2B). Confirming the deletion of TbZFP1, polymerase chain reaction (PCR) amplification of the TbZFP1 coding region from the null mutant lines resulted in no amplication, whereas a control gene (encoding TbZFP2; Hendriks *et al*. 2001) amplified at equivalent levels from both wild-type cells and the TbZFP1 null mutants (Fig. 2C). An analysis of the knockout lines in terms of growth, cell cycle status and morphology revealed no detectable difference from wild-type cells (data not shown). Although this was expected, given the lack of detectable TbZFP1 protein at this life cycle stage, this formally proved that TbZFP1 was not essential in bloodstream forms.

Trypanosome differentiation is compromised in the absence of TbZFP1

The generation of a TbZFP1 null mutant in bloodstream forms allowed investigation of the role of this gene during differentiation events. Progression from the bloodstream to procyclic forms can be dissected into a temporal order of events using markers for surface antigen exchange, cell cycle status and morphology (for review see Matthews, 1999). To investigate whether these events were perturbed in a TbZFP1 null genetic background, two independently derived bloodstream form TbZFP1 null mutants (null A, null B) were induced to differentiate to procyclic forms by the addition of 6 mM *cis*-aconitate and a drop in temperature to 27°C. Cells were harvested soon after the initiation of differentiation (6 h) and analysed for VSG loss and procyclin expression (Fig. 3A and B). Cells were then monitored after a further 72 h when those cells able to differentiate would be expected to have expressed available markers for procyclic forms. Figure 3 shows that VSG loss and EP procyclin gain were approximately equivalent in the wild-type cells and the TbZFP1 null mutants although in both cases the efficiency of these processes was quite low (only 50% of cells had gone on to express procyclin after 72 h, in contrast to > 90% cells in other populations routinely used in our laboratory). This low level of differentiation was observed consistently for the null mutants and the wild-type line from which they were directly derived and may result from long-term *in vitro* passage of these cells.

Although no consequence for cell differentiation was observed for surface antigen exchange a significant phenotype was detectable when cell morphogenesis was assayed. During differentiation between bloodstream and procyclic forms, the trypanosome mitochondrial genome, the kinetoplast, repositions from the very posterior end of a bloodstream cell to approximately half way between the nucleus and the posterior end in procyclic forms. This repositioning represents a change in distance between the kinetoplast and the posterior end of approximately 4 μm, this being a consequence of posterior microtubule extension (Matthews *et al*., 1995). When the TbZFP1 null mutants were examined for their ability to undergo kinetoplast repositioning, this capacity was found to be significantly compromised (Fig. 4A). At the start of differentiation both populations showed a mean kinetoplast to posterior end measurement of ~1 μm. This is in the normal range for a monomorphic bloodstream form population. However, when EP positive cells were examined 72 h post induction, the TbZFP1 null mutants had a mean kinetoplast to posterior measurement of 1.8 μm (null A = 1.80 μm; null B = 1.81 μm), whereas wild-type populations had a mean kinetoplast to posterior end dimension of 3.2 μm. Individual cell measurements are shown in Fig. 4A (left hand panel) and representative cell images are shown in Fig. 4B. This indicated that many of the TbZFP1 null mutant population had not undergone any

kinetoplast repositioning at all while a subset had repositioned the kinetoplast to a limited extent. This conclusion was highly reproducible and statistically significant ($P < 0.001$; see below) with each mutant generating similar results on each occasion. TbZFP1 null mutants therefore appear to complete normal cell surface antigen exchange but are specifically compromised in the ability to correctly change morphology and thus complete cellular differentiation.

To eliminate the possibility that the observed morphological phenotype was a consequence of a trait of the selected cell lines independent from TbZFP1 gene ablation, we generated bloodstream cell lines able to express a rescue copy of C-terminally Ty1-tagged TbZFP1 from the pHD430 expression vector (Biebinger *et al.*, 1997). Figure 4A (right hand panel) shows the relative kinetoplast-posterior dimension when wild-type cells, one TbZFP1 null mutant (null B) and the rescued cell lines were induced to differentiate to procyclic forms. Consistent with what was observed in the earlier experiments (Fig. 4A left panel), the null mutant showed a significantly reduced kinetoplast repositioning when compared with wild-type cells. Thus a one way analysis of variance (ANOVA) test demonstrated significant differences between the wild-type, null mutant and rescue populations ($P < 0.001$) whereas, a formal *post hoc* Tukey analysis demonstrated significant difference between the wild-type and null mutant population ($P < 0.001$) but not between the wild-type and rescue populations ($P = 0.11$). Interestingly, although significant differences were not observed between the wild-type population and the C-terminally tagged ZFP1 rescue population, individual cells were observed in the latter that not only underwent kinetoplast repositioning, but also apparently did so somewhat more effectively (Fig. 4A and B).

The repositioning of the kinetoplast during differentiation between bloodstream and procyclic forms is predominantly a consequence of outgrowth of the posterior end of the cell caused by posterior extension of the microtubule cytoskeleton (Matthews *et al.*, 1995). To formally confirm that the ablation of TbZFP1 perturbed this normal repositioning we analysed the outgrowth of the microtubule cytoskeleton by staining the cells with the antibody YL1/2, which is specific for dynamic microtubules in the cell and diagnostic for the normal events of kinetoplast repositioning. Figure 5 shows that the TbZFP1 null mutant line shows very limited staining of its cytoskeleton with YL1/2 during differentiation, consistent with the failure to fully reposition the kinetoplast. In contrast, analysis of the TbZFP1 rescue line revealed abundant staining with YL1/2 in those cells undergoing kinetoplast repositioning (arrowed in Fig. 5).

Combined these assays demonstrated that the normal repositioning of the kinetoplast during differentiation can be disrupted by TbZFP1 gene knockout, and that an epitope tagged copy of TbZFP1 can functionally rescue this.

Dependency relationships in the trypanosome developmental programme

During the synchronous differentiation of bloodstream stumpy forms to procyclic forms, the earliest event is the gain of procyclin, this being followed by loss of the VSG coat. Thereafter, kinetoplast repositioning occurs, this being coincident with DNA replication in the kinetoplast and nucleus. Following this, kinetoplast segregation occurs and then nuclear mitosis. An interesting question is whether this represents a linear pathway in which each event is dependent upon the completion of earlier events, or a branched pathway with some events being independent of others.

To address this question we examined whether TbZFP1 null mutants could segregate their kinetoplasts during differentiation, despite kinetoplast repositioning being compromised. This event normally occurs in a synchronous time course only after kinetoplast repositioning. Thus, the TbZFP1 null mutant population was examined for cells that had lost

their VSG coat and expressed EP and GPEET procyclin at 72 h after the initiation of differentiation with *cis* aconitate. These cells were selected because the regulation of these surface antigen markers is diagnostic for cells which have embarked on the differentiation pathway. This subpopulation was then searched for cells in which the kinetoplast remained closely adjacent to the posterior end of the cell, but in which kinetoplast segregation had occurred. Since kinetoplast repositioning precedes kinetoplast segregation, any such cells would have bypassed the need for morphological restructuring and progressed directly into the G2 phase (or beyond) of the cell cycle of the differentiated parasite. We found that EP procyclin +ve cells with segregated kinetoplasts positioned at the extreme posterior of the cell were observed in the differentiating TbZFP1 null population at a frequency of 18.5% (of 540 procyclin positive cells analysed, 100 had segregated kinetoplasts at 72 h after the initiation of differentiation). Of these the vast majority (99%) demonstrated aberrant positioning of the kinetoplasts (e.g. Figure 6A–E), cell nucleus (in 2K1N cells, e.g. Figure 6B and C) or nuclei (in 2K2N cells, e.g. Figure 6E and F) or other karyotypic defects (> 2 kinetoplasts or nuclei, for example) and there being a proportion (1%) of the procyclin positive cells which were anucleate cytoplasts (zoids). In contrast to the TbZFP1 null mutants, an analysis of the wild-type cell population at different time points (0 h, 6 h, 20 h and 72 h) after the initiation of differentiation revealed no cells with aberrantly positioned kinetoplasts (although a small proportion of zoids were observed).

We conclude that compromising kinetoplast positioning does not arrest the cells in differentiation; rather, the population successfully segregated their kinetoplasts, albeit with an accumulation of cell cycle defects. Clearly, two central events in trypanosome differentiation, kinetoplast repositioning and cell cycle progression, can be uncoupled by genetic ablation of TbZFP1.

Discussion

Differentiation of the African trypanosome from its bloodstream to procyclic form represents a well orchestrated series of events involving surface antigen exchange, morphological restructuring, cell cycle re-entry and metabolic adaptation to life in the fly. These processes are carefully coordinated, enabling the establishment of the appropriate cell architecture for proliferation as the procyclic form (Robinson *et al.*, 1995). Here we have examined the expression and consequences for development of the absence of a differentiation-enriched protein containing an RNA binding CCCH zinc finger motif.

TbZFP1 was originally identified in a screen for molecules transiently enriched during the synchronous differentiation of bloodstream stumpy forms to procyclic forms. Analysis of TbZFP1 expression revealed that it was transiently elevated between 2 h and 8 h after the initiation of the process, and then downregulated, reappearing in established procyclic forms. Analysis of the phenotypic consequences of TbZFP1 ablation indicates that the same processes occur in the same order in monomorphic forms, which differentiate asynchronously and with slower kinetics at the population level. Thus, kinetoplast repositioning was compromised, a process that initiates at around 6–8 h in a synchronous differentiation time course (Matthews *et al.*, 1995), coincident with the elevated expression of TbZFP1, and progresses until around 12 h, when TbZFP1 is downregulated (Hendriks *et al.*, 2001). This temporal relationship between TbZFP1 expression and kinetoplast repositioning, combined with the perturbation of this process upon TbZFP1 ablation, provides strong evidence for a causal relationship between these events. This is further strengthened by the observation that introduction of a rescue copy of TbZFP1 fully restores kinetoplast repositioning demonstrating that the expression and functionality of TbZFP1 is important in kinetoplast repositioning during differentiation.

The present study is the first to use genetic ablation to specifically disrupt a morphological event in differentiation and examine the effect on other differentiation events. First, surface antigen exchange occurred normally in the TbZFP1 mutants, these events being initiated before the normal expression of TbZFP1 in the mapped differentiation programme. In contrast, when cells which had failed in kinetoplast repositioning were studied with respect to their capacity for cell cycle progression, parasites that had successfully segregated their kinetoplasts were observed. This demonstrates that kinetoplast repositioning can be uncoupled from events of the cell cycle in differentiating cells, providing support for previous analyses using the DNA synthesis inhibitor aphidicolin during synchronous differentiation of stumpy forms to procyclic forms (Matthews and Gull, 1994). An alternative explanation for the observation that segregated kinetoplasts can be observed in differentiating cells is that monomorphic cell differentiation can be initiated in different windows of the cell cycle (Li *et al.*, 2003). In this scenario, G2 or post mitotic bloodstream cells (in which the segregating kinetoplasts are normally positioned at the extreme posterior of the cell) could initiate EP procyclin expression producing differentiating cells with apparently mispositioned kinetoplasts. Although an analysis of the wild-type population argues against this alternative explanation (we failed to identify procyclin-positive cells in which the segregated kinetoplasts were positioned at the cell posterior), the conclusion of the experiment would remain unchanged. Thus, once differentiation is underway there is not a dependency relationship between kinetoplast repositioning and cell cycle progression and the developmental pathway to procyclic forms is not strictly hierarchical (Fig. 7).

TbZFP1 is a member of a family of proteins which contain the CCCH domain. A second example, TbZFP2, is also implicated in the control of differentiation events. In that case, however, RNAi for TbZFP2 inhibited the initiation of differentiation (as assessed by the gain of EP procyclin), contrasting with TbZFP1 where surface antigen exchange occurs successfully, but kinetoplast repositioning is compromised. A stage-specific morphological phenotype (‘nozzle formation’) was also observed when TbZFP2 was ectopically over expressed either in differentiating cells or in established procyclic forms. Nozzle formation involved significant extension of the posterior end of the cell because of microtubule extension and the cells underwent cell division arrest in G1 (Hendriks *et al.*, 2001). One interpretation is that nozzle formation is an exaggerated manifestation of the same events that result in kinetoplast repositioning during differentiation to the procyclic forms. This implies that despite there being distinct consequences for differentiation when the expression of each is disrupted, TbZFP1 and 2 might be involved in related processes.

Posterior extension of the cytoskeleton has also been observed when a number of cell cycle regulators have been targeted by RNAi in *T. brucei*. For example, cyc2 ablation by RNAi generated cell-cycle arrested cells with an extended posterior (Hammarton *et al.*, 2004) as did targeted ablation of CRK1 + CRK2 in combination (Tu and Wang, 2005), although in the latter case cells with branched posterior extensions were also observed. Whilst these cell-cycle-related morphological phenotypes may be distinct from kinetoplast repositioning during differentiation, a link between TbZFP1 expression and procyclic cell-cycle control is feasible. Thus the apparent transient expression of TbZFP1 during differentiation between bloodstream stumpy and procyclic forms may represent periodic expression of this molecule during the synchronous cell cycle of the differentiating parasites, this coinciding with progression through the G1/S transition. Although this periodic expression would only be detected in the synchronized cell cycle of the differentiating population, it may be maintained in established and asynchronous procyclic populations which retain TbZFP1 expression. The inability to synchronize procyclic forms in culture, however, has limited our ability to test this possibility.

Although our results indicate that kinetoplast repositioning can be uncoupled from kinetoplast segregation during differentiation, there may still be possible downstream consequences for the cell. Indeed, the strict positioning of single copy organelles characteristic of procyclic forms make it unlikely that cells with aberrant morphology could undergo sustained cell division. Hence perturbing kinetoplast positioning through TbZFP1 ablation may result in cell cycle failure later in differentiation or in procyclic forms, perhaps explaining why viable TbZFP1 null mutants could not be generated in that stage. Alternatively, TbZFP1 may have a role in established procyclic forms distinct from that during differentiation. Thus, the phenotypes observed with TbZFP1 gene knockout may represent manifestation of a single effect (perturbed kinetoplast positioning disrupting the cell cycle during differentiation and in procyclic forms) or two distinct phenotypes, one affecting morphological restructuring during differentiation and the other affecting viability of procyclic forms by a related or unrelated mechanism.

The CCCH motif has been characterized in a number of eukaryotic proteins, frequently existing in tandem pairs. The prototype for proteins with this motif is the mammalian protein tristetraproline, a molecule involved in regulating mRNA stability (Blackshear, 2002). As further CCCH–containing proteins have been discovered, it has become clear that this motif is usually involved in RNA binding. Although this binding can be dependent on tandem copies of the zinger finger (Lai *et al.*, 2002), more recent studies demonstrate that one finger, as in TbZFP1, may be sufficient (Michel *et al.*, 2003). Although the known functions of CCCH-containing proteins are diverse, ranging from developmental control to protection against RNA viruses, the RNA targets for these proteins are frequently AU-rich element (ARE) containing transcripts (Lai *et al.*, 1999). As there is precedent for the regulation of ARE containing mRNAs in kinetoplastid development (Quijada *et al.*, 2002), it can be speculated that TbZFP1 influences differentiation through stabilizing or destabilizing such mRNAs or through modulating their cellular location or capacity for translation. Recently, however, an analysis of the *in vitro* RNA binding properties of a homologue of TbZFP1 in *Trypanosoma cruzi* has demonstrated a specificity for polyC (Morking *et al.*, 2004). This confirms the capacity for these molecules to bind RNA but suggests their targets may be a distinct subset of mRNAs from those containing AREs. Regardless of the specific targets, it is not surprising that differentiation regulators would operate at the RNA level because the organization of the trypanosome genome into polycistronic transcription units requires that most gene expression is regulated at the post-transcriptional level (Clayton, 2002). We are currently investigating the protein and RNA interactions of the small CCCH proteins in *T. brucei* in order to understand how these regulators of differentiation act at the molecular level.

Experimental procedures

Trypanosome cultures, transfection and differentiation

Monomorphic bloodstream form *T. brucei rhodesiense* EATRO 2340 were cultured in HMI-9 at 37°C, 5% CO_2 (Hirumi and Hirumi, 1989). Procyclic form *T. brucei* 427 forms were cultured in SDM-79 at 27°C (Brun and Schönenburger, 1979). Ectopic expression was carried out in the respective bloodstream or procyclic form *T. brucei* expressing the tetracycline repressor (Bs449 and Pc449).

Transfection of bloodstream and procyclic form parasites was carried out as described in (Hendriks *et al.*, 2001) using 10 μg of PCR amplified knockout targeting construct, or NotI (pHD451 or pHD430) linearized DNA, electroporated into 500 ml of cells at 4×10^7 cells ml^{-1}. Transfected cells were recovered overnight in 10 ml of HMI-9 at 37°C 5% CO_2 (bloodstream forms) or SDM-79 medium at 27°C (procyclic forms) before being diluted to 1×10^5 cells ml^{-1} and subjected to drug selection in 24 well plates. Drug concentrations used

for selection were: hygromycin (2 μg l^{-1} for bloodstream forms, 20–50 μg l^{-1} for procyclic forms), G418 (0.8–2.5 μg l^{-1} for bloodstream forms, 15 μg l^{-1} for procyclic forms), phleomycin (0.5–2.5 mg l^{-1} for bloodstream forms, 5 mg l^{-1} for procyclic forms) or blasticidin 20 μg l^{-1} (used for procyclic forms only). Selected cells were cloned by limiting dilution under drug selection. Differentiation of monomorphic bloodstream forms was carried out using 2 × 10^6 cells ml^{-1} of either *in vitro* or *in vivo* grown cells using 6 mM *cis*-aconitate and a drop in temperature to 27°C in HMI-9 (for *in vitro* grown cells) or SDM-79 media (for *in vivo* grown cells).

Plasmid constructs

The TbZFP1 coding region was PCR amplified using primers ZFP1.F and ZFP1.C-Tag or ZFP1.R (oligonucleotides used in this study are shown in Table 1). The TbZFP1 PCR product adds a Ty1 epitope tag C-terminal to the TbZFP1 coding region and was digested with HindIII/BglII for cloning into the trypanosome expression vectors pHD451 and pHD430 (Wirtz *et al.*, 1999) digested with HindIII/BamHI. These plasmids were then used to direct TbZFP1 transgene expression in either bloodstream or procyclic form parasites, which had been previously engineered to express the tetracycline repressor by stable transfection with the expression construct pHD449 (Wirtz *et al.*, 1999). This system is therefore tetracycline inducible and expressed proteins are detectable with the antibody BB2, which recognizes the Ty1 epitope (Bastin *et al.*, 1996).

Knockout constructs were made by PCR amplification of regions 5′ and 3′ of the TbZFP1 coding region, for cloning into two knockout vectors containing either a neomycin or hygromycin selectable marker. Specifically primers ZFP1 5′-Neo KO F/R and ZFP1 3′-Neo KO F/R were used to PCR amplify a TbZFP1 gene fragment 5′ and 3′ of the TbZFP1 coding region, and each product double digested with SacI/ NotI (5′ product) and KpnI/ XhoI (3′ product) and then cloned into the plasmid containing the tetracycline repressor and neomycin selection cassette. Similarly the primers ZFP1 5′ Hyg KO F/R and ZFP1 3′ Hyg KO F'R were used to PCR amplify TbZFP1 gene fragments 5′ and 3′ of the TbZFP1 coding region and each product double digested with SacI/NotI and KpnI/XhoI and cloned into the plasmid containing the hygromycin selection cassette. Precise details of all constructs used in this manuscript are available from the authors upon request.

Verification of TbZFP1 null mutants by PCR was performed using primers ZFP1 F/R (Table 1).

RNA and protein analysis

Trypanosome RNA was prepared using a Qiagen RNA-easy Kit (by the manufacturers method) and Northern blotting performed as described previously (Tasker *et al.*, 2000). Blots were hybridized at 60°C with riboprobes labelled with digoxygenin (Roche) and stringency washes were at 60°C, 0.1×SSC. Blot detection was by chemiluminescence using CDP-star as a reaction substrate.

Proteins were prepared as described in (Tasker *et al.*, 2000) and resolved on 15% polyacrylamide gels. Blotted proteins were incubated with either the anti-Ty1 epitope tag monoclonal antibody BB2 (diluted 1/20 from hybridoma culture supernatant), or a previously generated affinity purified rabbit- anti-TbZFP1 antibody diluted 1/200. Western blots were processed using alkaline phosphatase conjugated secondary antibody and visualized by chemiluminescence (Amersham).

Cell growth, morphometric analysis and immunofluorescence analysis

Transgenic cells were induced to express TbZFP1 by addition of tetracycline (1 μg l^{-1}) and growth monitored over a period of 1 week. At intervals approximately 2×10^6 cells were harvested, spread onto microscope slides and then air dried for 10 min. For *in vitro* differentiation experiments microscope slides were prepared as above. All slides were fixed in methanol at −20°C for at least 20 min. For immunofluorescence analysis slides were rehydrated in PBS, and then incubated (1 h) with primary antibodies for VSG (1 in 100), or EP procyclin (1 in 500) or YL1/2 (undiluted rat monoclonal hybridoma culture supernatant). Primary antibodies were removed by washing in PBS and secondary anti-mouse-TRITC (1 in 50), anti-rabbit-FITC (1 in 50) or anti-rat FITC (1 in 100) conjugates were applied (1 h). After washing cells in PBS, DAPI (4,6-diamidine-2,phenylindole) was applied for 5 min. Finally the cells were washed in PBS and mounted in MOWIOL containing phenylene diamine (PDA, 1 mg l^{-1}). Slides were examined on a Zeiss Axioscop 2 and images captured using Scion image 1.62. Kinetoplast repositioning assays were performed using the measuring tool on video captured cells in Scion image 1.62. Figures were processed using Adobe Photoshop 6.0.

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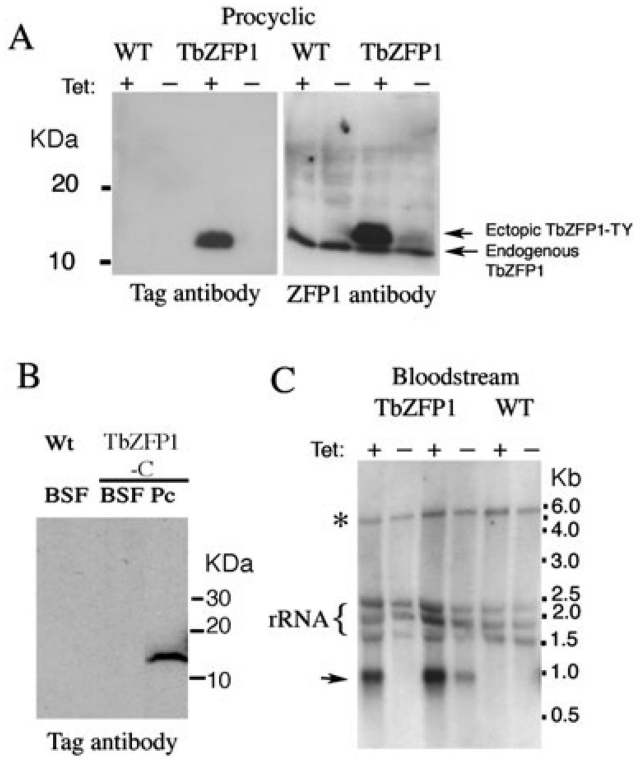

Fig. 1. TbZFP1 ectopic overexpression in Bloodstream and procyclic cells

A. The left panel shows a Western analysis of proteins derived from procyclic cells expressing ectopic TbZFP1, detected using the BB2 antibody to the Ty1 tag engineered into the protein. Cells were either induced (+) or uninduced (−) with tetracycline and the parental (Pc449; 'WT') strain is shown as a control. The right panel shows the same protein samples probed with an anti-peptide antibody to TbZFP1 and shows both the native molecule (lower band – all samples) and the transgenic protein (upper band).

B. Western analysis of wild-type bloodstream form cells ('Wt BSF') or bloodstream form cells transfected with a gene encoding a copy of TbZFP1 comprising a C-terminal Ty1 tag ('TbZFP1-C'). No tagged protein is detectable using the anti-tag antibody BB2. Procyclic cells containing the same expression construct are shown ('PC') as a control. Cells were grown in the presence of 1 μg ml^{-1} tetracycline to induce transgene expression.

C. Northern analysis of RNA samples taken from bloodstream form TbZFP1 expression cell lines induced (+) or uninduced (−) with tetracycline. Two clonal lines and the parental (Bs449 'WT') were probed with the TbZFP1 coding region. The asterisk indicates endogenous TbZFP1 mRNA, the arrowhead indicates the transgene-derived mRNA. Cross hybridizing rRNAs are also detected (labelled 'rRNA').

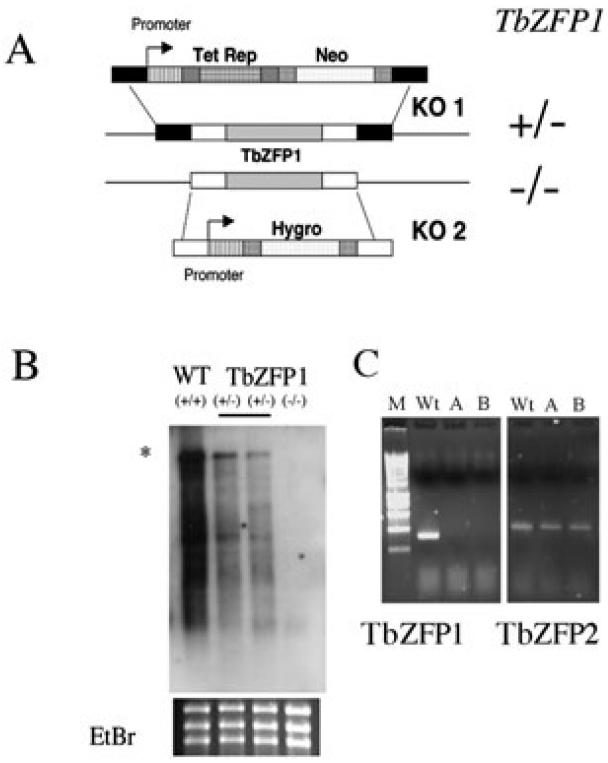

Fig. 2. Bloodstream TbZFP1 null mutants

A. Schematic diagram showing the strategy used to create TbZFP1 null mutants in bloodstream forms. The tetracycline repressor and neomycin cassette were used for the first knockout, and a set of targeting sequences internal to the first knockout targeting sequences were used for the hygromycin cassette for the second knockout. This ensured that the first integration could not be re-targeted during the second knockout. Note that each drug resistance cassette has also been engineered to contain an EP1 promoter (arrowhead), which is active in bloodstream forms, to ensure sufficient transcription of the drug resistance cassettes.

B. Northern analysis of bloodstream form cells deleted for *TbZFP1*. RNA samples from bloodstream forms were probed with a *TbZFP1* specific riboprobe. Wild-type (+/+), two single gene knockout cell lines (+/−) and a double knockout (−/−) mutant are shown. The blot has been overexposed to reveal the low level endogenous *TbZFP1* mRNA in the wild-type and single knockout lines and to confirm its absence in the null mutant line. The ethidium bromide image of the RNAs is shown as a loading control.

C. Verification of the deletion of TbZFP1 in the null mutant cells. In each case genomic DNA from either wild-type cells or the null mutant lines (Null A, Null B) was subjected to PCR amplification using primers specific for either the TbZFP1 coding region, or the coding region of TbZFP2 (Hendriks *et al.*, 2001), as a control. Each amplification reaction was run simultaneously.

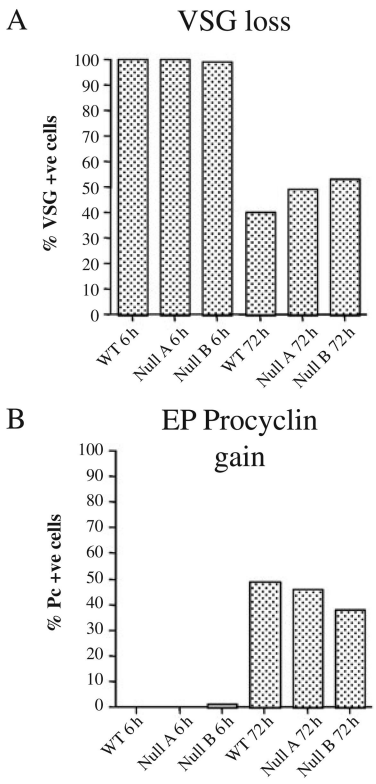

Fig. 3. Differentiation of Bloodstream TbZFP1 null mutants

A. Graph showing VSG loss for two TbZFP1 null mutants (null A and null B) and the wild-type (WT) cell line 6 h and 72 h after exposure to differentiation conditions.

B. Graph showing EP procyclin expression for the same cells as in A.

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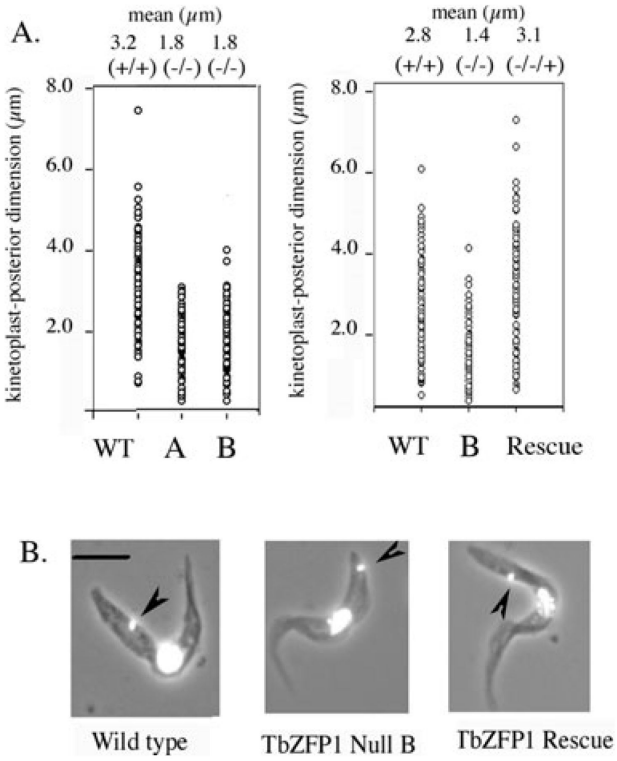

Fig. 4. Morphometric assay of differentiating TbZFP1 null mutants

A. Kinetoplast repositioning of wild-type cells (WT, ZFP+/+), two independently derived TbZFP1 null mutants (ZFP1 −/−; null A, B), and the null B mutant expressing a rescue copy of TbZFP1 (ZFP1 −/−/+). For the left hand panel, individual cell measurements are shown for the kinetoplast to posterior dimension 72 h after incubation in differentiation conditions. The right hand panel is a separate experiment in which the cell samples were taken 96 h after the initiation of differentiation. This time point was chosen to match the percentage differentiated cells (as assessed by expression of EP procyclin) seen in the experiment presented in the left panel, thereby allowing the relatively kinetoplast positioning in the respective populations to be more easily compared. Cells undergoing differentiation were identified by their expression of EP procyclin. Each open circle represents an individual cell measurement and 100 cells were measured for each time point. B. Images of the relative extent of kinetoplast repositioning in wild-type cells, the null B mutant and the null B mutant re-expressing a rescue copy of TbZFP1. In each case a phase-contrast-DAPI image is shown. The arrowhead marks the position of the kinetoplast. Scale bar = 8 μm.

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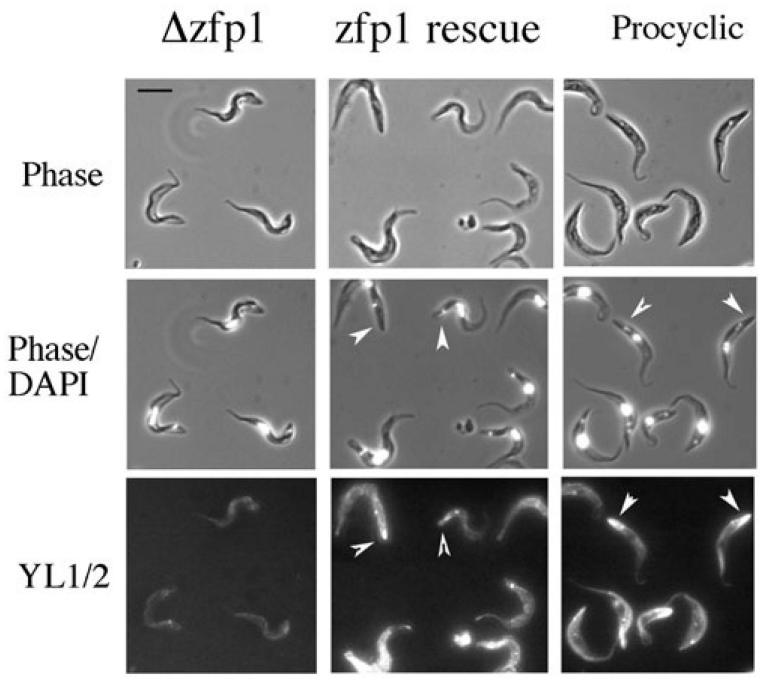

Fig. 5.

Absence of posterior microtubule extension on the TbZFP1 null mutant. Representative images are shown of the TbZFP1 null mutant cells and null mutants expressing an ectopic rescue copy of TbZFP1 at 96 h after the initiation of differentiation to procyclic forms. Procyclic form cells are also shown, as a control. In each case phase contrast images of cells are shown (‘Phase’) as are the same cells counterstained with DAPI to reveal the position of the kinetoplast (‘Phase DAPI’). The lower panels show the same fields stained with Yl1/2, which detects tyrosinated microtubules within the cell, this being diagnostic for posterior extension of the cytoskeleton. Example cells in which there is active posterior microtubule extension are highlighted with arrowheads. Bar = 15 μm.

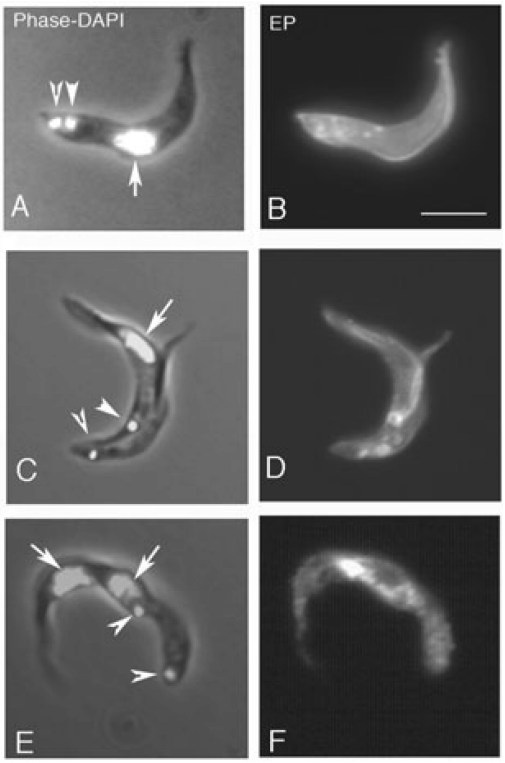

Fig. 6.

Cells progress into the cell cycle despite a failure in kinetoplast repositioning. Cells from the null B population are shown 96 h after being induced to differentiate to procyclic forms. In each case the left panel shows cells stained with DAPI to indicate that the kinetoplasts have segregated, despite being located at the extreme posterior of the cell. Kinetoplasts are labelled with an arrowhead; nuclei are labelled with an arrow. The right panel shows the same cells stained with EP procyclin, confirming that each cell is undergoing differentiation and is not merely a proliferating bloodstream form parasite.

A and B. Showing a cell where kinetoplast repositioning is initiating.

C and D. Showing a cell where the kinetoplasts are well segregated, but where one kinetoplast remains close to the posterior of the cell. This cell may also be undergoing an aberrant attempt at cytokinesis.

E and F. Show a cell where kinetoplast segregation is large and mitosis has occurred. Nonetheless one kinetoplast remains close to the posterior end of the cell. Bar = 10 μm.

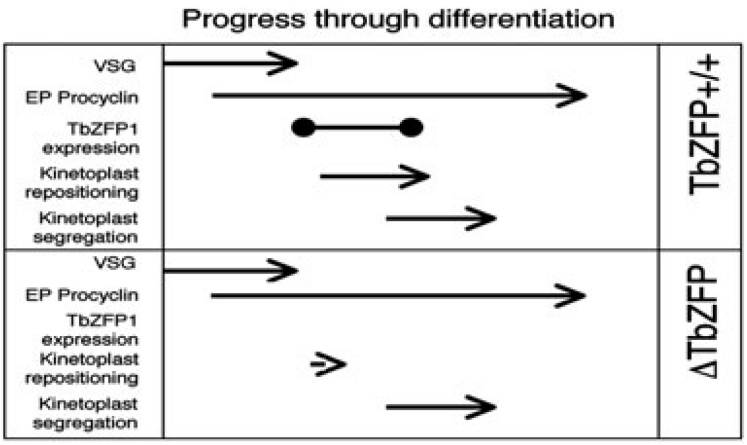

Fig. 7.

Schematic representation of the phenotypic consequences of TbZFP1 knockout in the context of the pathway of events during bloodstream stumpy-procyclic form differentiation. The expression profile of TbZFP1 is shown in the wild-type cells as a dumbbell.

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Table 1

Oligonucleotides used in this study.

ZFP1 F	CCC AAG CTT ATG CAC ATT TCA ACA CCC
ZFP1 R	CCA GAT CTG TCT CAA TCC TCC AAC CT
ZFP1 C- Tag	CCC AGA TCT TCA GTC AAG TGG ATC CTG GTT AGT ATG GAC CTC ATC CTC CAA CCT TAC GCT
ZFP1 5′-Neo KO F	GGG GTA CCG TTT TCC ATG TCA CTC AA
ZFP1 5′-Neo KO R	GCT CGA GGT GGG AAT GGA TAT CTC C
ZFP1 3′-Neo KO F	TGC GGC CGC GTG TAT TTG GAT TTG C
ZFP1 3′-Neo KO R	CGA GCT CCG TCT TGG TTT GTA GGA A
ZFP1 5′ Hyg KO F	CGA GCT CGA CCT CAA GCG ACC TTT T
ZFP1 5′ Hyg KO R	TGC GGC CGC TCC TTC CGG GTT ATC TCT TC
ZFP1 3′ Hyg KO F	GCT CGA GGA CAT TTG GAA AGG GGA A
ZFP1 3′ Hyg KO R	GGG GTA CCC ACA ACG CAC AAT TTC C